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PROCESS FOR CELL CULTURING BY CONTINUOUS PERFUSION

5   The present invention relates to perfusion culturing of cells.

The present invention discloses a process for the culturing of cells by perfusion culturing of a cell culture comprising cell culture medium and cells, wherein cell culture medium is added to the cell culture, wherein the cell culture is circulated over a filter module comprising hollow fibers resulting in an outflow of liquid having a lower cell 10   density than the cell culture and wherein the flow within the filter module is an alternating tangential flow.

It has surprisingly been found that by perfusion culturing of animal, in particular mammalian, cells or yeast cells according to the invention, extremely high viable cell densities can be obtained, whereas the cell culture further displays an extremely high 15   cell viability. Furthermore, it was found that the perfusion process of the invention leads to less cell aggregation in the culture, and even to a culture being a suspension of single cells without visible aggregates. This is a surprising finding because the use of low shear conditions, such as in perfusion cell culturing, typically does not lead to disaggregation of cells. Cell aggregation during perfusion cell culturing is 20   disadvantageous, because process control is more difficult, due to, for example, the heterogeneity in metabolic profiles of cells within the cell aggregates. This is especially troublesome if cells form aggregates of 5 cells or more and when the aggregates comprise in total 5 % or more of the total amount of cells.

A perfusion process is described in US 6,544,424. Although this document 25   mentions that this process may be used for perfusion culturing of animal cells, it does neither disclose nor suggest the extremely high cell densities found in the present invention. Furthermore, US 6,544,424 B1 discloses that the perfusion process could diminish the attachment and growth of an obstruction on the membrane surface of the hollow fibers, but it does neither disclose nor suggest that cells in the cell culture itself 30   would aggregate less.

Voisier et al. (Biotechnol. Bioeng. 82 (2003), 751-765) review various cell retention techniques in high-density perfusion culture of suspended mammalian cells. None of the reviewed cell retention systems is able to provide the extremely high viable cell densities combined with the extremely high cell viability, of the present invention.

35   Perfusion culturing of cells has its conventional meaning in the art, i.e. it means that during culturing cells are retained by a separation device in which there is an

outflow of liquid having a lower cell density than prior to separation and in which there is an inflow of the cell culture medium. In the process of the present invention, the separation device is a filter module comprising hollow fibers.

Perfusion culturing includes, but is not limited to continuous flow and semi-continuous flow, for example step-wise flow or staggered flow.

With the term "hollow fiber" is meant a tubular membrane. The internal diameter of the tube is preferably between 0.3 and 6.0 mm, more preferably between 0.5 and 3.0 mm, most preferably between 0.5 and 2.0 mm. Preferably, the mesh size in the membrane is chosen such that the size of the pores in the mesh is close to the diameter of the cells, ensuring a high retention of cells while cell debris can pass the filter. Preferably, the mesh size is between 3-30 μm.

Filter modules comprising hollow fibers are commercially available from for example General Electric (formerly Amersham).

With "alternating tangential flow within the filter module" is meant that there is one flow in the same direction as (i.e. tangential to) the membrane surfaces of the hollow fibers, which flow is going back and forth, and that there is another flow in a direction substantially perpendicular to said filter surface. Tangential flow can be achieved according to methods known to the person skilled in the art. For example, in US 6,544,424 it is described that alternating tangential flow can be achieved using one pump to circulate the cell culture over a filter module comprising hollow fibers and another pump to remove the liquid having a lower cell density than prior to the filter separation.

In the process of the invention, any type of cell culture medium suitable for the culturing of cells can in principle be used. Guidelines for choosing a cell culture medium and cell culture conditions are well known in the art and are for instance provided in Chapter 8 and 9 of Freshney, R. I. Culture of animal cells (a manual of basic techniques), 4th edition 2000, Wiley-Liss and in Doyle, A., Griffiths, J. B., Newell, D. G. Cell &Tissue culture: Laboratory Procedures 1993, John Wiley & Sons.

Generally, a cell culture medium for mammalian cells comprises salts, amino acids, vitamins, lipids, detergents, buffers, growth factors, hormones, cytokines, trace elements and carbohydrates. Examples of salts include magnesium salts, for example $MgCl_2.6H_2O$, $MgSO_4$ and $MgSO_4.7H_2O$ iron salts, for example $FeSO_4.7H_2O$, potassium salts, for example KH_2PO_4, KCl; sodium salts, for example NaH_2PO_4, Na_2HPO_4 and calcium salts, for example $CaCl_2.2H_2O$. Examples of amino acids are all 20 known proteinogenic amino acids, for example hystidine, glutamine, threonine, serine, methionine. Examples of vitamins include: ascorbate, biotin, choline.Cl, myo-inositol,

D-panthothenate, riboflavin. Examples of lipids include: fatty acids, for example linoleic acid and oleic acid; soy peptone and ethanol amine. Examples of detergents include Tween 80 and Pluronic F68. An example of a buffer is HEPES. Examples of growth factors/hormones/cytokines include IGF, hydrocortisone and (recombinant) insulin.

5 Examples of trace elements are known to the person skilled in the art and include Zn, Mg and Se. Examples of carbohydrates include glucose, fructose, galactose and pyruvate.

The pH, temperature, dissolved oxygen concentration and osmolarity of the cell culture medium are in principle not critical and depend on the type of cell chosen.

10 Preferably, the pH, temperature, dissolved oxygen concentration and osmolarity are chosen such that it is optimal for the growth and productivity of the cells. The person skilled in the art knows how to find the optimal pH, temperature, dissolved oxygen concentration and osmolarity for the perfusion culturing. Usually, the optimal pH is between 6.6 and 7.6, the optimal temperature between 30 and 39°C, the optimal

15 osmolarity between 260 and 400mOsm/kg.

Cells that are advantageously subjected to the process of the invention may be any cell type benefiting from this process, i.e. culturing to an extremely high viable cell density and an extremely high cell viability.

According to the process of the invention, an extremely high viable cell density

20 is a density of at least 80×10^6 cells per mL, preferably at least 100×10^6 cells per mL, more preferably at least 110×10^6 cells per mL, more preferably at least 120×10^6 cells per mL, more preferably at least 130×10^6 cells per mL, most preferably at least 140×10^6 cells per mL. Typically, a suitable upper limit in the cell density may lie around 500×10^6 cells per mL.

25 Surprisingly, the extremely high cell density of the process of the invention is accompanied by an extremly high cell viability. An extremely high cell viability is a viability of at least 90%, preferably at least 95%, more preferably at least 97%, most preferably at least 99%.

It is to be understood that the very high viable cell density and very high cell

30 viability are reached after a certain period of perfusion culturing, generally when the cells have reached a steady state, for mammalian cells typically 12 to 25 days after the initiation of perfusion culturing.

The process of the invention is suitable for culturing animal cells or yeast cells, especially for culturing mammalian cells.

35 The process of the invention is further especially suitable for culturing cells that easily or inherently form aggregates during culturing, especially during perfusion

culturing (so-called aggregating cells). Surprisingly, the process of the invention not only diminishes aggregate disposal on the filter membrane, but also diminishes aggregation of cells during the perfusion culture process, even aggregation of cells with an inherent tendency to form aggregates. The culturing of aggregating cells according to the invention results in a culture wherein aggregates of at least 5 cells comprise at the most 5 % of the total amount of cells, preferably at the most 4 %, more preferably at the most 3 %, even more preferably at the most 2 % of the total amount of cells. Especially preferably, the culturing of aggregating cells according to the invention results in a culture that is a real single cell suspension.

Aggregating cells are cells that form aggregates of at least 5 cells, the aggregates comprising in total at least 5 % of the total amount of cells. Preferably, the aggregates consist of at least 6, more preferably at least 7, even more preferably at least 8, even more preferably at least 9, even more preferably at least 10 cells. Preferably, the aggregates comprise in total at least 7%, more preferably at least 10 %, most preferably at least 15% of the total amount of cells.

Examples of mammalian cells include: CHO (Chinese Hamster Ovary) cells, hybridomas, BHK (Baby Hamster Kidney) cells, myeloma cells, human cells, for example HEK-293 cells, human lymphoblastoid cells, PER.C6® cells, mouse cells, for example NS0 cells. Examples of yeast cells include *Saccharomyces cerevisiae, Phaffia rhodozyma, Kluyveromyces lactis,* or yeast cells from the genus *Pichia* .

Preferably, mammalian cells are used, more preferably CHO, NS0, PER.C6® cells. Also preferably, cells known for their aggregating behaviour during culturing (aggregating cells) are used. Most preferably, PER.C6® cells are used.

Cell aggregation may for example be determined under a microscope.

The rate of addition of cell culture medium to the culture (the inflow rate or perfusion rate) influences the viability and the density of the cells.

In one embodiment of the invention, the cell culture medium is added at a perfusion rate according to the following formula 1:

Perfusion rate = SPR*total cell culture volume*viable cell density   (1)

wherein the perfusion rate is expressed in liters per day, wherein the SPR is the specific perfusion rate, i.e. the rate in which the cell culture medium is fed to the cell culture expressed as the volume of medium added per viable cell per time unit and wherein the viable cell density is the number of viable cells per unit of volume. The number of viable cells can be determined by the person skilled in the art, for example

via the trypan blue exclusion method.

The specific perfusion rate is preferably chosen between 0.01 and 0.3 nL/cell/day, more preferably between 0.01 and 0.2 nL/cell/day.

It may be of advantage to take into account additional parameters when adjusting the perfusion rate, for example the amount of glucose to be fed to the culture and/or the oxygen concentration. For example, for PER.C6® the glucose perfusion rate is preferably chosen between 3 and 20 mmoles/L, more preferably between 5 and 15 mmoles/L, as part of the medium perfusion rate.

A person skilled in the art knows how to determine the outflow rate. The outflow rate of the liquid is determined by the perfusion rate and is generally chosen at an equal value.

In one embodiment of the invention, the outflow liquid is substantially devoid of viable cells.

In another embodiment of the invention, biomass (i.e. cells in cell culture) is removed at least once from the cell culture and additional cell culture medium is added to the cell culture to compensate for the biomass removal. Biomass removal may lead to higher cell densities. Biomass may be removed continuously or step-wise.

In the step-wise approach, biomass is removed continuously for a defined time period. If a step-wise approach is used, biomass removal is preferably started just before or just after the cells have reached a steady state.

If a step-wise approach is used, a volume of biomass of preferably between 2 and 40 % of the working volume per day, more preferably between 5 and 30% of the working volume per day, even more preferably between 10 and 25% of the working volume per day is removed per biomass removal step.

With "working volume" is meant the total volume of the cell culture.

With "biomass removal step" is meant the time from the start to the stop of the biomass removal. If a continuous approach is used, the biomass is removed continuously until the end of the cell culturing. Preferably, the continuous removal of biomass is started just before or just after the cells have reached a steady state. Preferably, a volume of biomass is removed of between 2 and 40 % of the working volume per day, more preferably between 3 and 30% of the working volume per day, even more preferably between 4 and 15% of the working volume per day.

The addition of the additional cell culture medium is done to compensate for the biomass removal. The feed wherein additional cell culture medium is added to the cell culture may be merged into the perfusion feed, but may also be added in a separate feed. The person skilled in the art is aware how much additional cell culture medium is

needed to compensate for the biomass removal. Generally, the rate of addition of the additional cell culture medium to the cell culture will be the same as the biomass removal rate.

5 In yet another embodiment of the invention, a biological substance is produced by the cells. The biological substances that can suitably be produced in the perfusion culturing of the cell are in principle all biological substances that can be produced by animal, especially mammalian, and yeast cells, for example therapeutic and diagnostic proteins, such as monoclonal antibodies, growth factors or peptide hormones, enzymes, polynucleotides, such as viral vectors used in gene therapy, vaccines, etc.

10 In the perfusion culturing process of the invention, the outflow liquid will have a lower cell density but the same concentration of the biological substance than the liquid prior to separation.

Preferably, the process according to the invention is used for the production of a biopharmaceutical product, which is a biological substance with a medical

15 application. Examples of biopharmaceutical products are as follows (with examples of brand names of the corresponding biopharmaceutical product between brackets): Tenecteplase (TN Kase^{TM}), (recombinant) antihemophilic factor (ReFacto^{TM}), lymphoblastoid Interferon α-n1 (Wellferon^{TM}), (recombinant) Coagulation factor (NovoSeven^{TM}), Etanercept, (Enbrel^{TM}), Trastuzumab (Herceptin^{TM}), Infliximab

20 (Remicade^{TM}), Basiliximab (Simulect^{TM}), Daclizumab (Zenapaz^{TM}), (recombinant) Coagulation factor IX (Benefix^{TM}), erythropoietin alpha (Epogen®), G-CSF (Neupogen®Filgrastim), Interferon alpha-2b (Infergen®), recombinant insulin (Humulin®), Interferon beta 1a (Avonex®), Factor VIII (KoGENate®), Glucocerebrosidase (Cerezyme^{TM}), Interferon beta 1b (Betaseron®), TNF alpha

25 receptor (Enbrel®), Follicle stimulating hormone (Gonal-F®), Mab abcixmab (Synagis®, ReoPro®), Mab ritiximab (Rituxan®), tissue plasminogen activator (Activase ®, Actilyase®), human growth hormone (Protropin®, Norditropin®, GenoTropin^{TM}). Examples of polynucleotides with a possible medical application are gene therapeutic plasmid DNAs. Some gene therapeutic DNAs are presently tested in

30 clinical trials for their medical application. Examples of vaccines are live, oral, tetravalent Rotavirus vaccine (RotaShield^{TM}), rabies vaccine (RanAvert^{TM}), Hepatitis B vaccin (RECOMBIVAX HB®, Engerix®) and inactivated hepatitis A vaccine (VAQTA^{TM}).

The biological substance in the outflow may be further purified in so-called

35 downstream processing. Downstream processing usually comprises several purification steps in varying combinations and order. Examples of purification steps in

the downstream processing are separation steps (e.g. by affinity chromatography and/or ion exchange chromatography), steps for the concentration of the biological substance (e.g. by ultrafiltration or diafiltration), steps to exchange buffers and/or steps to remove or inactivate viruses (e.g. by virusfiltration, pH shift or solvent detergent treatment).

The invention will now be elucidated by way of the following examples, without however being limited thereto.

Example 1: Process Optimisation of the Human Cell Line PER.C6® for the production of Biopharmaceuticals

Introduction

A number of expression platforms now exist for the production of biopharmaceuticals. Most of the new products must choose a mammalian system due in main part to the glycosylation machinery which these cells contain and others lack. However to date the cell mass and resulting productivity of these cells is a factor of 10 –100 times less than a corresponding microbial system if these cells had the machinery to make such products.

A perfusion culture setup was developed for the PER.C6® cell line, a human cell line that possesses a number of features that makes it favourable for the production of biopharmaceuticals. A perfusion setup involves the separation of various components of the culture broth so that cells are retained, harvest is captured and medium refreshment occurs. The performance of a spinfilter, an acoustic device and an Alternating Tangential Flow (ATF) unit within a continuous perfusion culture of the PER.C6® cell line was assessed.

Materials & Methods

Cell line and maintenance : A PER.C6® cell line was used in this study that produces a human IgG. Cells were maintained in a serum free commercial medium (EX-CELL™ VPRO medium, JRH Biosciences), supplemented with 6 mM L-glutamine (Gibco). The PER.C6® cell line is a human embryonic cell-line immortalised with adenovirus type-5 (ad5) E1 gene using a phosphoglyceratekinase promoter.

Bioreactor Set-up: 1L and 4L working volume reactors (Applikon, Netherlands and

B.Braun, Germany) were used during this study. A Braun DCU3 controller (B. Braun, Germany) was used to operate the process at defined setpoints. Temperature was maintained at 36.5°C (range 35.5-37.5 °C). Dissolved oxygen concentration was controlled at 50% (range 40-60%) of air saturation by automatic adjustment of inlet gas
5   composition through the headspace and intermittent sparging through a microporous sparger. The pH setpoint was 7.1 (range 6.7-7.5) and controlled by the flow of CO_2 via the headspace. Cells were inoculated in the fermenter with an inoculum viable cell density range of 0.2-0.5 $*10^6$ cells/mL. Perfusion started at a viable cell density in the range of 1-3$*10^6$ cells/mL.

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Cell Retention: Cells were retained in the reactor using three different devices. First a spinfilter with a 10µm pore size (GKD, Düren Germany) was used. Secondly, a Biosep ADI1015 cell retention system and controller (AppliSens, the Netherlands) was used. Finally an ATF-4 control unit and housing with associate hollow fiber membrane
15 module (Refine Technology, USA) was assessed. The hollow fiber filter used was model CFP-2-E-8SIP (0.2 micron, Area: 4600cm^2, Amersham Bioscience obtained from Magellan instruments, USA). To maintain a constant culture volume a level sensor control loop was in operation.

20 Analytical methods: A cell count from the bioreactor was performed using the trypan blue exclusion method. The number of viable cells was determined as follows: An amount of cells stained with trypan blue was transferred to a Fuchs Rosenthal haemacytometer. The chamber of the haemacytometer was placed under a microscope and an appropriate number of boxes was counted. The viable cell density
25 was calculated using the following formula:

Viable cell density ($\times10^5$ cells/ml) = (A+B) x E/320   (2)

Wherein
30 A = number of non-stained cells in square A
B = number of non-stained cells in square B
E = dilution factor

Antibody concentration was determined by a analytical protein A column using a HPLC
35 with UV280nm absorption detection; the actual concentration was determined on basis of a calibration curve of a IgG1 reference standard.

Results

Perfusion Cultures

5   Results obtained with the above materials and methods are shown in Figures 1-6.

Legends to the Figures:

Figure 1: Viable cell density ($\times 10^6$ cells/ml) versus culture time (days) for two different continuous perfusion fermentations of an IgG1 producing PER.C6® clone using a

10   spinfilter separation device. Stirrer speed setting of the 1L Applikon fermenter was 100-150rpm. The perfusion runs were performed in 1L working volume. The specific perfusion rate (SPR) for both perfusion runs was 0.1-0.3 nL/cell/day. In both cases the perfusion runs had to be terminated because of spin filter clogging .

Figure 2: Growth of IgG1 producing PER.C6® cells in a continuous perfusion system

15   with an acoustic device as a cell retention system. Stirrer speed setting of the 1L Applikon fermenter was 100-150rpm. The settings used for the run/stop cycle were 300s forward and 4.5s backwards. During the run this was adapted to a 300s/3s cycle (day 15). The specific perfusion rate (SPR) for the perfusion run was between 0.1-0.3 nL/cell/day.

20   Figure 3: Growth of IgG1 producing PER.C6® cells in a continuous perfusion system with an ATF-4 unit as a cell retention system. The experiment was performed in a 4L Applikon fermenter. Setting for the stirrer speed was 125 rpm. The ATF-4 operated between 0.5 and 3 working volumes per day. The SPR was set at 0.03-0.08 nL/cell/day. The inset shows the high cell density of the culture, being completely

25   devoid of aggregating cells.

Figure 4: Productivity of IgG1 versus culture time (days) for two different continuous perfusion fermentations of a IgG1 producing PER.C6® clone using a spinfilter separation device.

Figure 5: Productivity of IgG1 producing PER.C6™ cells in a continuous perfusion

30   system with an acoustic device as a cell retention system.

Figure 6: Productivity of IgG1 producing PER.C6® cells in a continuous perfusion system with an ATF unit as a cell retention system.

Summary

See Table 1 for an overview of the data obtained for the different types of perfusion.

Table 1. Overview of the viable cell density, volumetric production rate (based on reactor volume) and the yield improvement of the perfusion runs using the three different retention devices. Batch and fed-batch results are added for comparison (data not shown).

Process	Max. Viable Cell Density (10^6 cells/mL)	Productivity	Yield (total amount of product produced) Improvement Factor
Batch	8-10	0.5 g/L	1
Fed-Batch	8-10	1.2 g/L	2.4
Continuous Perfusion			
Spin filter retention device	20-30	0.1-0.2 g/L/day	2.8-5.6
Acoustic retention device	20	0.6 g/L/day	16.8
ATF retention device	100	0.9 g/L/day	25.2

It can be concluded that continuous perfusion experiments using the ATF unit show significant potential to achieve very high cell densities and product concentrations (100×10^6 cells/mL and 0.9 g/L/day), while no aggregation of the PER.C6® cells was observed.

Example 2: Culturing of PER.C6® cells by perfusion.

Equipment: B.Braun fermenter control unit (Braun, Germany), 7L Braun vessel and headplate with associated pH, dissolved oxygen (DO) and level sensor probes (Braun, Germany), ATF-4 control unit and housing with associate hollow fiber membrane module (Refine Technology, USA).

Filter

Filter model: CFP-2-E-8SIP

Type: 0.2 micron

Area: 4600 cm^2

Amersham Bioscience

Working volume

Setpoint: 4.1 L

Range: 3.8 – 4.7 L

5   ATF settings

Parameter	Setpoint	Range
Pressure rising setpoint (psi)	Variable	2 - 4
Pressure rising flow (L/min)	3.2	2.5 – 4.0
Exhaust flow (L/min)	3.2	2.5 – 4.0
Exhaust time (s)	Variable	3 - 8
Pre-pressure (psi)	Variable	5 - 9

Bleed rate

No biomass removal was applied to this process.

10   Materials: 6 mM (final volume) L-glutamine (Gibco) in Ex-CELL^{TM} VPRO medium (JRH
Bioscience, USA), 12% Na_2CO_3 is used to control the pH.

Cell Lines and Culture Conditions

A PER.C6® cell line expressing a model IgG was investigated in this study. The

15   PER.C6® cell line is generated from retina-derived primary human cells. The PER.C6®
cell line is able to generate complete human monoclonal antibodies (including the
glycans) (ref 1, ref 2).

Cells were cultured in shaking Erlenymer flasks at 110 rpm and 36.5°C. The
headspace of these flasks was controlled using a mixture of 5% CO_2/Air.

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Ref 1: Jones, D. H., van Berkel, P. H. C., Logtenberg, T. and Bout, A., 2002, 'PER.C6
cell line for human antibody production', Gen. Eng. News 22, 50-54.

Ref 2: Jones, D. et al., 2003, 'High-level expression of recombinant IgG in the human
cell line PER.C6', Biotechnol. Prog. 19, 163-168.

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Operation of Fermenter

Cells were cultured in a fermenter where dissolved oxygen tension, pH, temperature
and agitation rate were controlled as detailed below.

Parameter	Setpoint	Range	
Temperature	36.5°C	35.5 – 37.5	
pH	> 6.7	7.5 –6.7 Active pH control using 12% Na_2CO_3 if pH<6.7	
DO	50%	40 –60%	
Agitation	100 – 300	Staged increase as viable cell density (VCD) increases;	
		VCD ($x10^6$ cells/ml)	Agitation (rpm)
		0.3 – 10	120
		10 –30	150
		30 –50	170
		50 –80	200
		80 – 100	230
		100 –120	260
		>120	300

Process Description:

Cells are inoculated in a fermenter with an inoculation viable cell density range of 0.2 – 0.5 x 10^6 cells/ml and a setpoint of 0.3 x 10^6 cells/ml. Perfusion is begun when the viable cell density > 2 x 10^6 cells /ml or at day 5 of the culture whichever is achieved first.

The perfusion rate is dependent on the cell density of the culture and the rates used are described in the table below. Both the flow rate and the dilution rate are adjusted as the cell density in the fermenter increases.

Perfusion rates utilized for culture of PER.C6® cells

Viable cell density ($x10^6$ cells/ml)	Specific perfusion rate (nl/cell/day)	Setpoint of specific perfusion rate (nl/cell/day)
Day 1 of perfusion	0.15 – 0.25	0.2
3 – 50	0.03 – 0.06	0.04
50 –80	0.025 – 0.035	0.03
>80	0.01 – 0.03	0.02

The actual data and results from this example (amongst others flow rates and specific perfusion rates used in this example) are shown in Table 2 below and in Figures 7 and 8.

Figure 7: Culture time (days) versus flow (L/day) and specific perfusion rate (SPR in nl/cell/day) for PER.C6® cells cultured using a perfusion process.

Figure 8: Viable cell density and cell viability using the procedure described in example 2.

Table 2. Raw data obtained for example 2

Time	flow rate (FR)	dilution rate (D)	Specific perfusion rate (SPR)	Viable count (VC)	Viability	product concentration	Specific production rate of IgG1	Volumetric production rate
day	L/day	working volume/day	nL/cell.day	10^6/mL	%	g/L	pg/(cell.day)	g/L.day
0	0.00	0.00	0.00	0.6	90	0.012	NA	NA
1	0.00	0.00	0.00	0.3	77	0.008	NA	0.000
2	0.00	0.00	0.00	0.3	73	0.008	0.0	0.000
3	0.00	0.00	0.00	0.5	80	0.013	12.1	0.000
4	0.00	0.00	0.00	0.9	87	0.019	9.3	0.000
5	0.00	0.00	0.00	1.4	92	0.033	12.0	0.000
6	2.39	0.52	0.20	2.6	95	0.035	5.5	0.009
7	1.06	0.24	0.05	4.9	95	0.054	9.5	0.017
8	2.70	0.57	0.08	7.3	97	0.073	7.2	0.026
9	2.60	0.57	0.05	12.3	97	0.067	3.5	0.040
10	4.29	0.95	0.05	18.6	97	0.115	7.8	0.069
11	5.40	1.20	0.04	26.9	97	0.140	7.0	0.137
12	6.80	1.48	0.05	31.8	96	0.127	5.6	0.179
13	7.39	1.68	0.04	41.4	99	0.129	5.6	0.202
14	8.28	1.88	0.04	44.3	98	0.139	5.8	0.238
15	10.26	2.33	0.03	68.3	98	0.116	4.4	0.269
16	10.70	2.43	0.03	86.1	99	0.151	4.6	0.318
17	12.10	2.63	0.03	80.3	98	0.163	4.9	0.397
18	11.83	2.57	0.02	112.3	98	0.292	7.6	0.592
19	12.50	2.78	0.02	123.0	99	0.291	6.6	0.780
20	12.09	2.57	0.02	126.0	99	0.293	6.3	0.781
21	11.91	2.59	0.02	135.0	98	0.332	6.5	0.806
22	13.70	2.98	0.02	127.5	97	0.395	8.2	1.012
23	10.00	2.17	0.02	128.5	95	0.470	9.3	1.114